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Effects of double and triple bonds on the spatial representations of odorants in the rat olfactory bulb

Brett A. Johnson^{*,1}, Joan Ong¹, Kaman Lee¹, Sabrina L. Ho¹, Spart Arguello¹, and Michael Leon¹

¹Department of Neurobiology and Behavior, University of California, Irvine, Irvine, CA 92697-4550

Abstract

Many naturally occurring volatile chemicals that are detected through the sense of smell contain unsaturated (double or triple) carbon-carbon bonds. These bonds can impact odors perceived by humans, yet in a prior study of unsaturated hydrocarbons we found only very minor effects of unsaturated bonds. In the present study, we tested the possibility that unsaturated bonds affect the recognition of oxygen-containing functional groups, because humans perceive odor differences between such molecules. We therefore compared spatial activity patterns across the entire glomerular layer of the rat olfactory bulb evoked by oxygen-containing odorants differing systematically in the presence, position, number, and stereochemistry of unsaturated bonds. We quantified activity patterns by mapping [¹⁴C]2-deoxyglucose uptake into anatomically standardized data matrices, which we compared statistically. We found that the presence and number of unsaturated bonds consistently affected activity patterns, with the largest effect related to the presence of a triple bond. Effects of bond saturation included a loss of activity in glomeruli strongly activated by the corresponding saturated odorants, and/or the presence of activity in areas not stimulated by the corresponding saturated compounds. The position of double bonds also affected patterns of activity, but *cis* versus *trans* configuration had no measurable impact in all five sets of stereoisomers we studied. These results simultaneously indicate the importance of interactions between carbon-carbon bond types and functional groups in the neural coding of odorant chemical information and highlight the emerging concept that the rat olfactory system is more sensitive to certain types of chemical differences than others.

Keywords

2-deoxyglucose; odors; imaging techniques; mapping

We have been describing systematic differences in the spatial activity patterns in the glomerular layer of the rat olfactory bulb that are evoked by odorants that differ systematically in chemical structure (e.g., carbon number, functional group, open versus closed-chain structures, enantiomers, and stereoisomerism involving different branching and functional group position; Johnson et al., 1998, 1999, 2002, 2004, 2005a,b, 2006; Linster et al., 2001; Johnson and Leon 2000a,b; Ho et al., 2006a,b; Farahbod et al., 2006). Individual glomeruli in the bulb appear to receive convergent input from nasal olfactory sensory neurons that express the same single odorant receptor gene, and the location of glomeruli associated with the same odorant receptor are remarkably consistent from animal to animal (Mombaerts et al., 1996). By mapping the responses of glomeruli in the bulb to systematically related odorants, we therefore have been

*Correspondence to: Brett A. Johnson, PhD Dept. of Neurobiology & Behavior 2205 McGaugh Hall University of California, Irvine Irvine, CA 92697-4550 telephone: (949)824-7303 fax: (949)824-2447 email: bajohnso@uci.edu

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able to identify a number of molecular features that are particularly important in determining the response specificity of particular parts of the glomerular layer and their related receptors (Leon and Johnson, 2003). These responses typically involve glomeruli that are clustered with respect to odorant specificity, and the clusters often contain systematic arrangements of glomeruli that are related to particular chemical properties such as molecular length (Johnson et al., 1998, 1999, 2004; Johnson and Leon, 2000b; Ho et al., 2006a). The resulting overall patterns of activity appear to be relevant to perception in that the degree of similarity in the patterns is predictive of the degree of similarity in perceived odor for a variety of series of closely related odorant chemicals (Linster et al., 2001; Cleland et al., 2002; Schaefer et al., 2002; Ho et al., 2006a,b).

As part of a study of systematically differing odorant hydrocarbon structures, we recently included hydrocarbons differing in the presence, number, position, and stereochemistry of double bonds and triple bonds (Ho et al., 2006b), which are functional groups that can participate as acceptors in hydrogen bonding. Replacing a single carbon-carbon bond with a double or triple bond reduces flexibility, decreases hydrophobicity, and results in different shapes compared to the corresponding saturated compounds, all aspects of ligands that we would expect to lead to differences in receptor binding. We therefore were surprised that the presence and characteristics of these bonds did not have very much of an effect on the spatial representations of hydrocarbon odorants (Ho et al., 2006b). The main effects we detected were a decrease in the activation of ventral modules with decreasing bond saturation, especially when the double or triple bonds were present in the middle of the open-chained compounds. Given a similar effect of the presence of methyl substitution in the middle of these same open-chained compounds, the most likely explanation of this phenomenon seemed to be a relatively non-specific disruption of receptor binding to the longest available straight hydrocarbon chain in the odorant molecule (Ho et al., 2006b).

However, the presence of double and triple bonds affects the perception of odors of a number of odorants as judged by humans (Table 1; Arctander, 1994; Hatanaka et al., 1992). In some cases, these effects appear to be subtle, such as the differences between 3,7-dimethyloctanol (single bonded) and citronellol (double bonded), whereas the effects appear more dramatic in other cases, such as the difference between fruity and green odor descriptors associated with the single-bonded methyl octanoate and the triple-bonded methyl 2-octynoate (Table 1). Differences in the position, number, and stereochemistry of double bonds also can change minor odor notes or modifying descriptors such as “sweet” or “dry” that are applied by humans (Table 1).

Many of the odorants displaying differential odors with respect to bond saturation and stereoisomerism have oxygen-containing functional groups in addition to the double or triple bonds. It seemed possible that some of the effects of bond saturation on perceived odor for these odorants (as compared to the hydrocarbons we studied previously) might involve an interaction between the recognition of oxygen-containing functional groups by odorant receptors and differences in the overall odorant molecule imparted by the unsaturated bond.

To explore further the possible effects of double and triple bonds, we therefore investigated a number of sets of odorants with oxygen-containing functional groups that differed in the presence, type, number, location, and stereoisomerism of double and triple bonds (Table 2). This set of chemicals includes odorants that have been characterized as having differences in perceived odor (Table 1). We tested the hypothesis that these odorants would have differences in their evoked activity patterns despite the relative lack of effects involving hydrocarbons differing in the same parameters (Ho et al., 2006b).

MATERIALS AND METHODS

Odorants

The 28 odorants used in this study are listed in Table 2, which includes both our own preferred name and the unique Chemical Abstract Services registry number associated with each compound. Structural drawings illustrating the molecules are provided within the individual figures showing patterns of odorant-evoked [^{14}C]2-dexoyglucose (2-DG) uptake. Table 2 also indicates vendors and the purities they reported for their products. Minor impurities can have a substantial impact on patterns of 2-DG uptake, leading to differences in the patterns evoked by the same odorant obtained from different sources (Ho et al., 2006a), as we report further in our experiments on methyl octanoate in the present paper. Caution therefore is warranted in interpreting comparisons of any particular pair of odorant-evoked activity patterns. The relationships between neural responses and odorant chemistry might be determined more accurately by studying a very large number of odorants, a very large number of examples of each type of systematic difference in odorant chemistry, and multiple sources of each odorant chemical, which is what we have done in the present study.

Table 2 also indicates the dilutions of each odorant, as well as the resulting parts per million in air calculated from the vapor pressures of those compounds that we were able to volatilize from neat liquid. Values of vapor pressure were medians of all unique values taken from two Internet databases (PhysProp Database from Syracuse Research Corporation at <http://www.syrres.com/esc/physdemo.htm> and the Chemical and Physical Properties Database from the Pennsylvania Department of Environmental Protection at http://www.dep.state.pa.us/physicalproperties/CPP_Search.htm), which were accessed between the dates of October, 2003 and August, 2004, depending on the odorants, and two chemical modeling software packages (Molecular Modeling Pro version 3.14, ChemSW, Fairfield, CA and ChemDraw Ultra version 6.0, CambridgeSoft, Cambridge, MA). For some chemicals (e.g., methyl 2-octynoate), only a single estimated value of vapor pressure was available, which decreased our confidence in these values. For this reason, we used fixed dilutions rather than fixed values of vapor concentration in some experiments. Because three odorants were either solid (*trans,trans*-2,4-hexadien-1-ol), or affordable in only small quantities (nonadialenal isomers), we first diluted them in white, light mineral oil (Fisher Scientific), and therefore we were unable to estimate their final vapor concentrations.

The exposures comprising this study were organized into several independently executed experiments involving related odorant chemicals. Table 2 displays the number of animals tested with a given exposure condition in a given experiment. The data matrices from these animals were averaged together after subtracting the average response of littermates exposed to clean air to generate the uptake pattern representing the odorant exposure.

Odorant Exposures

The UC Irvine Institutional Animal Care and Use Committee (IACUC) approved all procedures involving rats. Rats aged between postnatal days 18 and 22 were given subcutaneous injections of [^{14}C]2-DG (1.6 $\mu\text{L/g}$, 0.1 mCi/mL, 52 mCi/mmol, Sigma Chemical Company, St. Louis) immediately before being placed into a 2-liter Mason jar that was modified to allow odorant exposures. Without delay, volatilized odorant began entering the chamber through a hole bored in the lid while a second hole in the lid provided for odorant exhaust.

Research-grade, high-purity nitrogen gas was bubbled through the liquid odorant preparation in a gas-washing bottle to volatilize the odorants. The odorized nitrogen vapor then was mixed with ultra-zero grade air prior to entering the exposure chamber. For neat odorants, the flow rate into the chamber was 2 L/min, whereas for odorants suspended in mineral oil, the flow

rate was 1 L/min. The odor delivery system was equilibrated for at least 15 minutes prior to an exposure. All tubing, regulators, and connectors were constructed from inert materials such as brass, Kynar, or Teflon[®]. Each set of tubing was used with only one odorant. For each study, one rat per litter was exposed to vehicle vapor as a blank condition before any of the odorant exposures. When an experiment employed an odorant suspended in mineral oil, mineral oil alone served as the blank condition, whereas nitrogen was passed through an empty gas-washing bottle as a blank when the experiment involved neat odorant liquids.

Exposures continued for 45 minutes and were terminated by removing and killing the rat by decapitation, followed by rapid dissection of the brain and olfactory bulbs. Brains were frozen at about -45°C in 2-methylbutane cooled on dry ice. Brains were stored at -80°C until sectioning in a cryostat.

Data analysis

Tissue sectioning, histological processing, imaging, and data analysis were performed as described previously in greater detail (Johnson et al., 1999; 2004). Briefly, uptake of radiolabeled 2-DG in the glomerular layer was measured using autoradiographic images of 20- μm coronal sections that were separated by 120 μm . Images of adjacent cresyl violet-stained sections were overlain on the autoradiograph images to locate the glomerular layer. Sampling was standardized with reference both to anatomical landmarks encountered along the anterior-posterior dimension in the stained sections and to a set of polar grids positioned on each section relative to the location of the glomerular layer. The grids were chosen on the basis of the position of the section between the anterior-posterior landmarks. Data were converted from grayscale units to units of nCi/mg tissue by comparison to radioactivity standards exposed to each sheet of autoradiography film. Data from individual sections were concatenated into matrices that then were further standardized by compression or by insertion of new data to equalize the number of sections between pairs of landmarks.

The matrices from the left and right bulbs of each animal were averaged, and the units were converted to a ratio of glomerular layer uptake to uptake measured from standardized portions of the subependymal zone of the bulb. For each experiment, the matrices of all clean air-exposed animals were averaged and subtracted from the data for each matrix. Units in each matrix then were converted to z-scores relative to the mean and standard deviation of values across that matrix. .

Statistical analyses for the data matrices in each study first involved calculating Pearson correlation coefficients for each pair of individual animal data matrices, regardless of the odorant used in the exposure. To calculate these correlations, corresponding cells of the z-score matrices serve as x-y pairs. The resulting correlation coefficients were combined into a matrix that then was used as input data for a principal components analysis. If different animals exposed to the same odorant yielded similar patterns of uptake, and if different odorants produced different patterns of uptake, loadings on the extracted factors from the principal components analysis would differ with respect to the odorant. Such odorant-dependent clustering of activity patterns was visualized in the current study by using two-dimensional plots of two of the extracted factors showing odorant-dependent differences. The statistical significance of these differences in factor loadings was evaluated by using t-tests or analyses of variance (one-way or two-way), depending on the experimental design.

Within each experiment, the z-score matrices involving the same odorant exposure condition were averaged together and plotted as color-coded contour charts. We prefer to orient these charts in a ventral-centered format to marginalize missing values along the dorsal surface. These missing values reflect the occasional loss of tissue during sectioning. Other illustrations of the same data, including dorsal-centered charts and 3-dimensional, rotatable images of the

activity patterns can be seen both at <http://leonsever.bio.uci.edu> and at [URL to be completed by publisher].

RESULTS

Effects of double and triple bonds on representations of methyl esters and acids

As a first test of the effect of double and triple bonds, we exposed rats to methyl octanoate (single bonds), methyl *trans*-2-octenoate (double bond), and methyl 2-octynoate (triple bond), which differ in the presence of unsaturated bonds very near a methyl ester functional group (Fig. 1A). Humans regard the three odorants as differing in perceived odor (Table 1; Arctander, 1994). Because we had preliminary evidence that the activity patterns evoked by methyl octanoate might differ depending on the particular preparation used, we also compared three different sources of methyl octanoate in this experiment.

As shown in Figure 1A (open arrowheads), all three preparations of methyl octanoate activated paired anterior regions that are known to respond to methyl and ethyl esters (Johnson et al., 1998, 1999, 2002, 2004, 2005a,b; Johnson and Leon 2000a,b). In contrast, the double-bonded odorant methyl *trans*-2-octenoate did not activate these regions very strongly, and the triple-bonded methyl 2-octynoate did not activate them at all (Fig. 1A). Both methyl octanoate and methyl *trans*-2-octenoate, but not methyl 2-octynoate, stimulated other regions of the bulb (Fig. 1A, outlined areas) that overlap with part of the representations of methyl and ethyl esters of similar carbon number (Johnson et al., 2004, 2005b). In addition to this region of activity, methyl *trans*-2-octenoate stimulated slightly more posterior glomeruli that were not activated by methyl octanoate (Fig. 1A, white arrows), whereas methyl 2-octynoate activated extremely posterior and ventral glomeruli that were not activated by either of the other two odorants (Fig. 1A, black arrows).

There were minor differences in the average uptake patterns evoked by the methyl octanoate preparations from different sources. For example, the preparation from Alfa Aesar evoked additional small areas of uptake in the posterior third of the midlateral and midmedial aspects of the bulb, resulting in yellow spots in the color-coded contour charts that were not apparent for the other two preparations (Fig. 1A, small black arrowheads). These areas of uptake that are detected in one 99%-pure preparation, but not in others, are more likely to represent responses to minor contaminants rather than part of the response to the label compound, which is the same for all preparations.

Statistical analysis confirmed a significant difference in the activity patterns evoked by the three methyl esters differing in bond saturation. In order to compare these patterns, we used principal components analysis, a standard multivariate statistical tool that addresses the multidimensional relationships between complex data sets such as these arrays of 2-DG uptake (Royet et al., 1987; Farahbod et al., 2006; Ho et al., 2006a,b). In this analysis, we first calculated a Pearson correlation coefficient for each pair of patterns in the study, using corresponding locations in each pair of z-score standardized arrays as x-y pairs. Because there were five animals exposed to each of the five odorants in this particular experiment, we thereby obtained a 25-by-25 matrix of correlation coefficients representing all of the pairs of the individual animals. This correlation matrix was used as input data for principal components analysis, which reduces the multidimensional relationships between the patterns into one-dimensional, arbitrary “factors”. The “first” factor is the one that explains the greatest proportion of the variance between the data matrices.

Figure 2A shows a plot of the first two factors identified by principal components analysis for the experiment whose average patterns are shown in Figure 1A. Points located near one another in this plot represent relatively similar patterns, whereas points that are further removed from

one another represent relatively dissimilar patterns. Therefore, the relative clustering of the points representing individual animals exposed to different sources of methyl octanoate indicates that the different preparations of methyl octanoate yielded overall patterns that are more similar to one another than are the patterns evoked by three odorants differing in bond saturation. Also, the plot shows that the patterns for the single-bonded methyl octanoate are more similar to the patterns for the double-bonded methyl *trans*-2-octenoate than to the patterns for the triple-bonded methyl 2-octynoate (Fig. 2A).

The first factor extracted by principal components analysis accounted for 41% of the variance between the individual animal activity patterns. By comparing the loadings on this factor across odorants using ANOVA, we were able to determine that the different odorants produced significantly different overall patterns of uptake ($F_{4,20} = 42.5$, $P < 0.0001$). Post-hoc Fisher PLSD tests on factor one loadings indicated significant differences between methyl 2-octynoate and every preparation of methyl octanoate, between methyl *trans*-2-octenoate and every preparation of methyl octanoate, and between methyl 2-octynoate and methyl *trans*-2-octenoate. However, factor one loadings were not significantly different among the different preparations of methyl octanoate. Differences among the odorants also were statistically significant for both the second factor (13% of the variance, $F_{4,20} = 93.9$, $P < 0.0001$) and the third factor (5% of the variance, $F_{4,20} = 9.2$, $P = 0.0002$) that were extracted by principal components analysis. Post-hoc Fisher PLSD tests on factor two loadings not only found significant differences among the three odorants that differed in bond saturation, but also found differences between methyl octanoate obtained from Aldrich and methyl octanoate obtained from Alfa Aesar.

Methyl esters can become hydrolyzed easily into corresponding carboxylic acids, which might explain, at least in part, their activation of anterior modules that are stimulated intensely by the acids. We therefore tested the possibility that a lesser acid contamination in the triple-bonded methyl 2-octynoate preparation might have been responsible for the absence of activation of the anterior modules. To this end, we purchased fresh methyl octanoate and methyl 2-octynoate as well as octanoic acid and 2-octynoic acid, the molecule resulting from hydrolysis of methyl 2-octynoate, and we exposed additional groups of rats to these odorants.

As shown in Figure 1B, our results with methyl octanoate and methyl 2-octynoate were replicated in this second experiment. As expected, octanoic acid stimulated the anterior acid-responsive modules intensely (Fig. 1B, open arrowheads), yet the triple bonded 2-octynoic acid did not stimulate this module, activating instead the posterior modules activated by the triple-bonded methyl 2-octynoate (Fig. 1B, arrows). These data suggest that the olfactory system may recognize the triple bond in these molecules as a distinct molecular feature.

Statistical analysis of the patterns underlying Figure 1B showed that the odorant-evoked patterns in this study were very robust and reproducible across animals. The first two factors extracted by principal components analysis were significantly different with respect to both bond type and functional group as judged by a two-way ANOVA conducted on the factor loadings, and loadings on the third factor were significantly related to functional group (Table 3). A plot of factor 1 versus factor 3 extracted by principal components analysis illustrates the dramatic odorant-dependent statistical clustering of the patterns (Fig. 2B).

The fact that triple-bonded 2-octynoic acid did not stimulate the otherwise acid-responsive region of the bulb suggests that the presence of a such a bond in proximity to either the acid or methyl ester group may disrupt the recognition of these compounds by odorant receptors involved in the anterior responses. It is very unlikely that different levels of acid contaminants can explain the evoked pattern differences between the methyl esters differing in bond saturation.

Effects of double bonds on representations of longer alcohols

To test the impact of double bonds distant from the functional group, we used a series of 10-carbon alcohols. Two of these molecules, 1-decanol and 9-decen-1-ol, are straight chained, but the latter has a double bond that is far removed from the alcohol functional group. The other two odorants, 3,7-dimethyloctan-1-ol and citronellol (3,7-dimethyl-6-octen-1-ol), are branched, and they also differ in that the latter has a double bond far removed from the alcohol functional group, while the former is single bonded. The average patterns of 2-DG uptake evoked by these four odorants are shown in Figure 3A. The underlying data matrices were subjected to principal components analysis followed by two-way ANOVA (branching versus bond type) on the first three factor loadings. The third factor significantly separated the odorants on the basis of both branching and bond type (Fig. 4A, Table 4). This analysis indicated that the differences in the four activity patterns shown in Figure 3A were statistically significant.

Inspection of the average 2-DG uptake charts showed that while the saturated odorants 1-decanol and 3,7-dimethyloctanol both stimulated uptake in the areas of the bulb that typically respond to primary alcohols (Fig. 3A, outlined regions), the double-bonded odorants did not activate these regions, despite the fact that the alcohol functional group was far removed from the double bonds in these molecules. These data suggest that the double bond interferes with the binding of these alcohols even at a considerable distance from the functional group.

Effects of double bonds on representations of cyclic hydrocarbons

Although we previously found that aliphatic hydrocarbons differing in bond saturation differed only slightly in their evoked glomerular activity patterns, we also wanted to test the effects of double bonds in cyclic hydrocarbons, because the constraints on their conformation already imposed by the ring structure suggest that the presence of double bonds might have less effect on the overall shape of the molecules than it would on aliphatic structures. Given the minimal effect on molecular rigidity, these cyclic molecules also may allow us to determine if there is any response potentially attributable to the change in hydrophobicity or electronic properties of the bonds themselves. To this end, we conducted an experiment in which we compared the glomerular responses of the odorants cyclooctene (with a single double bond) and 1,5-cyclooctadiene (with two double bonds) using our standard statistical analyses. We also compared these patterns less formally to those evoked by cyclooctane (single bonds) in a separate experiment (Fig. 3B).

Casual inspection suggested that the overall average activity patterns were quite similar for the three cyclic hydrocarbons that differed in the presence and number of double bonds. All three compounds activated areas typical for aliphatic and alicyclic hydrocarbons (Ho et al., 2006a,b; Johnson et al., 2006a). However, statistical analysis indicated that loadings on the second factor extracted by principal components analysis differed significantly between cyclooctene and 1,5-cyclooctadiene (Fig. 4B; $T = 2.65$, $P = 0.029$), suggesting that differences in electronic properties of odorants differing in the number of double bonds might still impact the activity patterns.

Effects of double bonds on representations of six-carbon alcohols

To test odorants differing in the location and stereochemistry of double bonds, we used a set of six-carbon alcohols that also included odorants differing in the presence and number of double bonds (Fig. 5). These odorants each have unique odor descriptions, most of which are related to green grass or foliage (Table 1; Arctander, 1994). Inspection of the average activity patterns suggested that most of the patterns evoked by double-bonded odorants differed in the posterior regions from the pattern evoked by the saturated odorant 1-hexanol (Fig. 5, in and around the outlined region). To determine if these localized differences were reliable across individual animals, we compared activity at different locations across the bulb by using a set

of standard “modules” that we have used in previous work to describe differences between odorants differing in functional group and hydrocarbon structure (Johnson et al., 2002). We determined the average z-score value within each of the 30 modules (Fig. 6) in each animal, and we then performed an ANOVA on each module to determine the statistical significance across odorants. Because of the high probability of a spurious positive outcome when making 30 different comparisons, we corrected the significance values by using a false discovery rate analysis (Curran-Everett, 2000). The asterisks in the upper left panel of Figure 6 indicate the three posterior modules that were found to be significantly different ($P < 0.05$) across odorants in this analysis. Figure 6 also shows the results of t-tests between 1-hexanol and each of the alcohols possessing a single double bond. Posterior modules consistently differed most reliably in these pair-wise comparisons.

To address the statistical significance of any differences in overall patterns, we performed principal components analysis using all individual patterns evoked by 1-hexanol, along with the patterns evoked by double-bonded odorants: *trans*-2-hexen-1-ol (double bond in the second position), *cis*-2-hexen-1-ol (double bond in the second position), *trans*-3-hexen-1-ol (double bond in the third position), *cis*-3-hexen-1-ol (double bond in the third position), and 5-hexen-1-ol (double bond in the fifth position). The odorant *trans,trans*-2,4-hexadien-1-ol (sorbic alcohol) was eliminated from this analysis because it was presented as a suspension in mineral oil (Table 2), with subtraction of a different blank, which can complicate the interpretation of any quantitative difference in patterns. The first and second factors, accounting for 40% and 9% of the variance, respectively, were found not to be significantly different with respect to odorant. However, the third factor, accounting for 5.6% of the variance, did differ significantly across odorants (ANOVA $F_{5,16} = 4.8$, $P = 0.007$) (Fig. 7).

Post-hoc Fisher PLSD tests identified differences between 1-hexanol and three other odorants (*cis*-2-hexen-1-ol, *trans*-3-hexen-1-ol, and 5-hexen-1-ol), indicating that the presence of a double bond significantly influenced evoked activity patterns. The odorant 5-hexen-1-ol also was found to be significantly different from *trans*-2-hexen-1-ol and *cis*-3-hexen-1-ol, suggesting that the position of the double bond can also influence activity patterns. Inspection of the average activity patterns evoked by these odorants indicated that the odorants with double bonds at positions 2 or 3 evoked more activity in mid-lateral and mid-medial regions of the bulb (Fig. 5, outlined areas) than did either 1-hexanol or 5-hexen-1-ol, whereas 5-hexen-1-ol evoked more 2DG uptake in posterior, ventral regions than did the other odorants (Fig. 5, solid black arrows). With the exception of *trans,trans*-2,4-hexadien-1-ol, which possesses two double bonds, all of these alcohols stimulated a pair of glomerular clusters within regions responding to numerous other primary alcohols (Fig. 5, open arrowheads). These data suggest that both the number and the position of the double bonds affect glomerular activity.

To specifically address the possible impact of stereoconfiguration at double bonds, we performed another principal components analysis involving only the *cis*- and *trans*-stereoisomers of 2- and 3-hexenol. Factor loadings then were subjected to a two-way ANOVA (stereochemistry versus bond position). There were no significant differences across odorants on any of the extracted factors, suggesting that the stereochemistry at the double bond had a minimal effect on the overall activity patterns, despite the fact that humans discriminate these odors (Laska, 2005) and despite the small differences that appear to be present in the average charts (Fig. 5).

Effect of the stereochemistry at double bonds on odorant spatial representations

Our experiments with six-carbon alcohols (Fig. 5) did not reveal any pattern differences between molecules differing in the stereochemistry at the double bonds. To explore the generality of this phenomenon, we investigated three additional pairs of odorants containing *cis* versus *trans* stereoconfigurations.

In one experiment, we exposed rats to the alcohols geraniol (*cis* configuration) and nerol (*trans*), as well as to the corresponding acetates (Fig. 8). The stereoisomers of these odorants are reported by humans to have distinguishable odors, as are the odorants with distinct functional groups (Table 1; Arctander, 1994). For all four odorants, the peak responses occurred in midlateral and midmedial parts of the glomerular layer (Fig. 8, open arrowheads). To test for significant differences among the overall patterns, we performed principal components analysis using the data matrix of pair-wise Pearson correlation coefficients between all of the individual z-score patterns in the study. In a subsequent two-way ANOVA (functional group versus stereochemistry) the first two extracted factors were found to differ significantly in loadings with respect to functional group (Table 5, Fig. 9). In contrast, none of the three extracted factors differed significantly with respect to either stereochemistry or the interaction between functional group and stereochemistry, suggesting that *cis* versus *trans* stereoisomerism was not a major factor in the spatial representations of these molecules. Inspection of the average uptake patterns evoked by the alcohols compared to the acetates (Fig. 8) did not reveal any obviously unique areas of response that would explain the statistical difference in the patterns. Rather, the relative intensity of the uptake in the midlateral and midmedial regions was consistently greater for the acetates than for the alcohols, which likely underlies the statistical significance in these analyses.

In a second experiment, we compared the *trans,trans* isomer of 2,6-nonadienal to the *trans,cis* isomer (Fig. 8). These stereoisomers also have been described by humans as eliciting distinct odor perceptions (Table 1; Arctander, 1994). As shown in Figure 8, bottom panels, both odorants stimulated uptake in ventral regions similar to those activated by other aldehydes (solid arrowheads). To test for significant differences in overall activity patterns between the two odorants, we performed a principal components analysis on pair-wise correlation matrices involving the individual animals. Then, t-tests were used to determine if loadings on the three extracted factors were significantly different between the two stereoisomers. None of the factors were found to differ with respect to stereochemistry (factor 1, 56.3% of variance, $T = 2.13$; factor 2, 10.1% of variance, $T = 0.007$; factor 3, 8.9% of variance, $T = 1.37$).

Together, the results of our experiments with the 2-hexenols, 3-hexenols, geranyl versus neryl compounds, and the 2,6-nonadienals, representing five pairs of stereoisomers, suggest that the stereochemistry at double bonds is not apparently important in determining the overall spatial patterns of 2-DG uptake in response to odorants.

DISCUSSION

Consistent effects of presence and number of unsaturated bonds

We have found that the presence and/or number of double or triple bonds had effects on spatial activity patterns for every one of the six different base structures investigated (methyl octanoate, octanoic acid, hexanol, decanol, 3,7-dimethyloctanol, and cyclooctane). Unsaturated bonds change the orientation of hydrocarbon chains in odorant molecules compared to the saturated compounds, and they render the molecules more rigid due to the absence of rotation around the bond. Because the electrons involved in the unsaturated bonds are not shared with hydrogen atoms, the unsaturated molecules have fewer hydrogen atoms available for hydrophobic van der Waal's interactions. The electrons in double and triple bonds also can themselves participate as acceptors in the formation of hydrogen bonds with other molecules, potentially including odorant receptors or water molecules. Any or all of these properties could be important in altering the set of receptors that interact with the unsaturated odorant as opposed to the corresponding saturated molecule, and thereby in changing the set of glomeruli responding to the odorant.

Neither 2-octynoic acid nor methyl 2-octynoate activated the anterior modules that were activated by the corresponding single-bonded molecules octanoic acid and methyl octanoate. These anterior modules have been activated by most acids and either methyl or ethyl esters in our previous studies (Johnson et al., 1998, 1999, 2002, 2004, 2005a,b; Johnson and Leon, 2000a,b). The absence of activity in the triple-bonded odorants indicates that the receptors responding to the single-bonded acids and esters are not able to bind to and/or be activated by the triple-bonded compounds. This response pattern constitutes a clear case of interaction between separate odorant molecular features to determine the overall activity pattern evoked by an odorant (Johnson et al., 2005b).

The triple bond in these molecules is very near the functional groups that have been associated with responses in this part of the glomerular layer. Any of the aforementioned changes in chemistry easily might have been responsible for the striking failure of the triple-bonded odorants to activate the receptors typically activated by modules with a specific functional group. For example, the linear stretch of hydrocarbon in these molecules may be incompatible with the shape of the binding site, or the absence of hydrogen atoms at carbons 2 and 3 may preclude binding to critical amino acid side chains, and/or the electronegative nature of the triple bond may cause it to be repelled by some oxygen-containing amino acid functional group in or near the receptor binding site.

Interference by a triple bond at position 2 also has been observed for the interaction between an eight-carbon aldehyde and the rat I7 odorant receptor, which tolerates a double bond in this position (Araneda et al., 2000). The I7 receptor is involved in a projection to glomeruli more ventral and posterior than the ones responding to the methyl ester and acid in the present study (Johnson et al., 2004), and the I7 receptor does not respond well to octanoic acid (Araneda et al., 2000), indicating that we are not simply characterizing the same receptor in a different way. Rather, the similarity in the degree of impact of a triple bond on these two different responses suggests that odorant receptors in general may be highly altered in their efficacy by odorant triple bonds.

A similar effect was observed for the double-bonded odorants 9-decen-1-ol and citronellol, which failed to activate glomerular modules that were stimulated by the corresponding saturated alcohols decanol and 3,7-dimethyloctanol. Most other primary alcohols also activated these anterior, dorsal modules in our previous studies (Johnson and Leon, 2000a; Johnson et al., 2002, 2004, 2005a,b). Because the double bonds in 9-decen-1-ol and citronellol are so remote from the alcohol functional group that is associated with activity in these modules, we were surprised by the large effect that the double bonds had on this activity. The effect of the double bonds in these 10-carbon odorants was especially unexpected given the observation that double bonds in multiple positions nearer the alcohol functional group in six-carbon primary alcohols did not interfere with the activation of glomeruli in the same region of the bulb. One obvious possibility is that the double bonds in the 10-carbon odorants specifically interact with more distant parts of the receptors that prevent the receptor from becoming activated despite the binding of the alcohol hydroxyl group. Again, this pattern of response represents a clear case of interaction between separate odorant molecular features in determining the overall spatial activity pattern evoked by the odorant.

The additional double bond in 1,5-cyclooctadiene compared to cyclooctene was associated with a statistically different evoked pattern of 2-DG uptake. The relatively rigid cyclic structures of both odorants would tend to reduce any difference in flexibility or shape caused by the additional double bond in 1,5-cyclooctadiene, increasing the chance that the differences in pattern between the two odorants would be related to differences in hydrophobicity or hydrogen bond acceptance.

Finally, the presence of a double bond in the series of six-carbon alcohols had a different effect than in the other studies. Instead of interfering with the activation of functional group-dependent glomerular modules, the double bonds appeared to be associated with the activation of glomeruli that were not activated by the saturated odorant 1-hexanol (Fig. 5, arrows and outlined areas), suggesting specific recognition of the double-bonded molecules by distinct sets of odorant receptors involved in projections to different bulbar regions.

In a study that involved screening sensory neurons expressing different receptors capable of interacting with octanal, several types of neurons were found to discriminate between different eight-carbon aldehydes on the basis of the presence and number of double bonds (Araneda et al., 2004). The odorants 1-hexanol and *cis*-3-hexen-1-ol as well as the odorants hexanal and *trans*-2-hexenal also were found to activate localized glomeruli differentially in optical imaging studies in rats (Uchida et al., 2000; Takahashi et al., 2004). These findings are in full agreement with our consistent identification of pattern differences for odorants differing in the presence and number of these bonds.

Effect of position

In the experiment involving six-carbon alcohols, we investigated three different positions of a double bond. We found that the pattern of 2-DG uptake evoked by 5-hexenol differed from the patterns evoked by 2- and 3-hexenols, which did not differ significantly from each other. The fact that the overall patterns were more similar for odorants having double bonds in more similar positions is an example of a global chemotopic organization similar to what we observed with isomers differing in the position of a functional group, where more similar positions also yielded more similar patterns (Johnson et al., 2005a). The systematic effect of double bond position in hexenols is fully consistent with a similar systematic effect of difference in bond position on the ability of human subjects to discriminate the odors of these molecules (Laska, 2005).

Consistent absence of effect of stereochemistry around double bonds

We studied five pairs of *cis* versus *trans* stereoisomers and did not once find a significant difference attributable to stereoconfiguration about the double bond, despite the fact that other significant differences were found within many of the same studies and despite the fact that the odors have been reported to be different for humans (Table 1; Arctander, 1994; Laska, 2005). These observations further highlight our recent recognition that the olfactory system does not function for general-purpose, high-resolution chemical analysis, but rather has apparently evolved for detecting particular odorants and chemical differences of greater importance to a particular species (Ho et al., 2006b; Johnson et al., 2006).

Nevertheless, the differences in odor of these stereoisomers as assessed by humans are commonly quite subtle. The principal descriptor (e.g., “green” or “rosy”) is commonly shared, while an adjective regarding this descriptor (e.g., “less fruity” or “sweeter”) may be different (Table 1; Arctander, 1994). Indeed, it seems possible that the pure compounds might not be easily discriminable by untrained people or animals. It may even be the case that discrimination depends on the detection of differential minor impurities that always exist at some level in any compound. Also, despite the similarities in overall activity patterns evoked by stereoisomers found using 2-DG uptake, there may be differences in the activation of individual glomeruli that are not apparent in our data due to variance in the exact positions of individual glomeruli across different animals.

Conclusions

Double and triple bonds can have major effects on odorant-evoked activity patterns in molecules with oxygen-containing functional groups. These effects can include the disruption

of activity in modules related to an odorant's oxygen-containing functional group. Therefore, interactions between double and triple bonds and responses to other parts of the molecule might influence activity patterns much more than in hydrocarbons that differ in carbon-carbon bond type but have no other functional group (Ho et al., 2006b). Conversely, stereoconfiguration about the double bond is less crucial to the overall activity pattern.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Anatomically standardized contour charts showing relative 2-DG uptake across the entire glomerular layer. Patterns represent averages of z-score standardized data matrices from several rats exposed to the same odorant condition. The orientation of the charts is shown at bottom right. The key to the color assignments is shown at center right. **A:** Patterns evoked by methyl ester odorants differing in the presence of single, double, or triple bonds at the 2-position. **B:** Patterns evoked in a second experiment by methyl ester and carboxylic acid odorants differing in the presence of single or triple bonds at the 2-position. Single-bonded odorants, but not double- or triple-bonded odorants, activated dorsal, anterior glomerular clusters previously reported to respond to methyl esters and acids (open arrowheads). Single- and double-bonded odorants both stimulated glomerular clusters previously found to be activated by numerous aliphatic compounds of this size (outlined areas). In addition, methyl *trans*-2-octenoate activated glomeruli located slightly more posteriorly (white arrows). Triple-bonded odorants stimulated posterior, ventral glomerular clusters (solid arrows) that were not stimulated by single or double-bonded odorants. Finally, one preparation of methyl octanoate had additional activity in a small area that was not activated by the other two preparations (small, black arrowheads), probably indicating the presence of a minor impurity stimulating glomeruli in this region.

Fig. 2.

Scatter plots of factors extracted by principal components analysis of the individual activity patterns involved in Figure 1. For each independent experiment, individual z-score-standardized uptake matrices were subjected to pair-wise Pearson correlation analysis. The resulting matrix of correlation coefficients was used as input data for a principal components analysis. Each point in the scatter plots represents an individual animal's pattern of 2-DG uptake. Relative clustering of points indicates overall similarity of pattern. **A:** Plot of factor 1 versus factor 2 extracted by principal components analysis of the experiment involving three methyl ester odorants differing in carbon-carbon bond type as well as three different preparations of methyl octanoate (Fig. 1A). Subsequent analyses revealed that both factor 1

and factor 2 contained significant information regarding odorant identity. **B:** Plot of factor 1 versus factor 3 extracted by principal components analysis of the experiment involving single or double bonds in methyl esters and carboxylic acids (Fig. 1B). Factor 1 was found to contain significant information regarding both bond type and functional group, while factor 3 was found to differ with respect to functional group only (Table 3).

Fig. 3. Anatomically standardized contour charts showing relative 2-DG uptake across the entire glomerular layer. Orientation and color scales are as described for Figure 1. **A:** Averaged z score patterns for rats exposed to straight-chained or branched ten-carbon alcohols differing in the presence of a double bond far removed from the alcohol group. The outlined area indicates a glomerular cluster that is activated by the single-bonded odorants, but not the double bonded odorants. **B:** Averaged z score patterns for rats exposed in a different experiment to cyclic, eight-carbon hydrocarbons differing in the number of double bonds.

Fig. 4.

Scatter plots of factors extracted by principal components analysis of the individual activity patterns involved in Figure 3. **A:** Plot of factors 1 and 3 from the experiment involving ten-carbon alcohols differing in branching and in the presence of double bonds (Fig. 3A). Subsequent analysis indicated that the third factor contained significant information regarding both branching and bond type (Table 4). **B:** Plot of factors 1 and 2 from the experiment involving cyclic, eight-carbon hydrocarbons differing in the number of double bonds (Fig. 3B). The second factor was significantly different with respect to number of double bonds.

Fig. 5.

Anatomically standardized contour charts showing relative 2-DG uptake across the entire glomerular layer in response to a series of six-carbon alcohols differing in the presence, number, position, and stereochemistry of double bonds. Orientation and color scales are as described for Figure 1. Open arrowheads indicate glomerular clusters commonly stimulated by primary alcohols that are also stimulated by all of the odorants in this series except for the odorant with two double bonds. Solid, black arrows indicate glomerular clusters that seem to be best stimulated by odorants having double bonds furthest from the alcohol group. The outlined area encloses glomeruli that also appear to be differentially stimulated by the different odorants.

Fig. 6.

Diagram of modular analyses of significant differences among six-carbon alcohols differing in the presence of one double bond. Average 2-DG uptake was determined in individual animals in 30 separate domains used in past studies to describe differences in activity evoked by odorants (lower right panel; Johnson et al., 2002). These values then were compared across odorants by using an ANOVA (upper left panel). The P values from the individual comparisons are indicated by differential shading of the modules according to the key at upper right. After the ANOVA, statistical significance was further explored by using false discovery rate analysis to correct for the multiple comparisons; asterisks in the upper left panel indicate the modules that were found to be significantly different at $P < 0.05$ in this corrected analysis. Also shown are diagrams of the P values obtained in each of five sets of t-tests comparing individual double-bonded odorants and 1-hexanol.

Fig. 7.

Scatter plot of factors 1 and 3 extracted by principal components analysis of the individual activity patterns evoked by six-carbon alcohols in Figure 5. Subsequent analysis indicated that the third factor contained significant information regarding odorant identity.

Fig. 8.

Anatomically standardized contour charts showing relative 2-DG uptake across the entire glomerular layer in response to three pairs of odorants differing in stereoconfiguration around a double bond. Orientation and color scales are as described for Figure 1. Open arrowheads show an activated glomerular cluster located in a similar position for isomers of both geraniol and geranyl acetate. The esters geranyl acetate and neryl acetate stimulated this area to a relatively higher level than did the corresponding alcohols. The solid arrowheads indicate a large glomerular cluster activated by both stereoisomers of nonadienal. This area also is stimulated by other aldehydes of similar carbon number.

Fig. 9. Scatter plot of factors 1 and 2 extracted by principal components analysis of the individual activity patterns evoked by isomers of geraniol and geranyl acetate (Fig. 8). Both factors were found to contain significant information about functional group, but not about stereoconfiguration (Table 5).

Table 1

Double and triple bonds affect odor descriptors that are applied to odorants.

Chemical difference	Odorants	Odor descriptors (Arctander, 1994)
Presence of double or triple bond	Methyl octanoate Methyl trans-2-octenoate Methyl 2-octynoate	Powerful, winey-fruity, orange-like Fruity-green, foliage Very powerful, penetrating vegetable-green foliage
Presence of double bond	1-Decanol 9-Decen-1-ol	Sweet, slightly fatty-oily, waxy, floral, rosy, lily, orange blossom Powerful, fatty-oily, waxy-rosy
Presence of double bond	3,7-Dimethyloctan-1-ol Citronellol	Waxy, dry-rosy Fresh, rosy
Presence, position, number, and stereochemistry of double bond	1-Hexanol <i>cis</i> -3-Hexen-1-ol <i>trans</i> -2-Hexen-1-ol <i>trans</i> -3-Hexen-1-ol <i>trans,trans</i> -2,4-Hexadien-1-ol	Somewhat "chemical"-winey, slightly fatty and fruity Powerful and intensely green, grassy (more grassy-green and foliage- green, less fruity than <i>trans</i> -2-hexen-1-ol) Powerful, fruity-green, slightly caramellic-fruity, chrysanthemum foliage or wine leaves (sweeter, more winey-leafy than <i>cis</i> -3-hexen-1-ol) Intensely green, but rather "bitter"-foliage-like, somewhat fatty (more chrysanthemum-like and less fruity than its two isomers) Sweet-oily, green, somewhat grassy-weedy, but quite refreshing Mild and sweet, floral, rose-type, warm and yet slightly dry undertones
Stereochemistry of double bond	Geraniol Nerol	Sweet-rosy, refreshing and "wet" seashore
Stereochemistry of double bond	Geranyl acetate Neryl acetate	Sweet, fruity, winey-green "fermented apple"-like, overall very pleasant Very sweet, fruity-floral, raspberry-rose, refreshing
Stereochemistry of double bond	<i>trans,trans</i> -2,6-Nonadienal <i>trans,cis</i> -2,6-Nonadienal	Extremely powerful, diffusive green-vegetable (sweeter, creamier and more attractive, natural-oily note) Extremely powerful, diffusive green-vegetable (more dry and sharp)

Table 2

Odorant exposures in this study¹.

Odorant	CAS#	Vendor	Purity (%)	Solvent	Solvent dilution	Air dilution	ppm	n
Methyl octanoate	111-11-5	Acros	99	None	None	1/8	49	10 ²
Methyl octanoate	111-11-5	Aldrich	99	None	None	1/8	49	5
Methyl octanoate	111-11-5	Alfa Aesar	99	None	None	1/8	49	5
Methyl <i>trans</i> -2-octenoate	7367-81-9	Alfa Aesar	99	None	None	1/8	39	5
Methyl 2-octynoate	111-12-6	Alfa Aesar	99	None	None	1/8	11	5
Methyl 2-octynoate	111-12-6	Aldrich	99	None	None	1/8	11	5
Octanoic acid	124-07-2	Sigma	> 99	None	None	1/8	1.7	5
2-Octynoic acid	5663-96-7	Aldrich	98	None	None	1/8	18	5
1-Decanol	112-30-1	Acros	> 96	None	None	1/8	2.5	5
9-Decen-1-ol	13019-22-2	Acros	99	None	None	1/57	2.5	5
3,7-Dimethyloctan-1-ol	106-21-8	Aldrich	> 98	None	None	1/8	2.5	5
Citronellol	106-22-9	Acros	95	None	None	1/36	2.5	5
Cyclooctane	292-64-8	Alfa Aesar	> 99	None	None	1/8	1200	4
Cyclooctene	931-88-4	Acros	95	None	None	1/8	924	5
1,5-Cyclooctadiene	111-78-4	Aldrich	99	None	None	1/8	715	5
1-Hexanol	111-27-3	Acros	99	None	None	1/12	100	3
<i>cis</i> -2-Hexen-1-ol	928-94-9	Acros	95	None	None	1/10	100	4
<i>trans</i> -2-Hexen-1-ol	928-95-0	Acros	96	None	None	1/41	100	4
<i>cis</i> -3-Hexen-1-ol	928-96-1	Acros	98	None	None	1/10	100	3
<i>trans</i> -3-Hexen-1-ol	928-97-2	Acros	95	None	None	1/41	100	4
5-Hexen-1-ol	821-41-0	Acros	99	None	None	1/41	100	4
<i>trans,trans</i> -2,4-Hexadien-1-ol	17102-64-6	Fluka	95	Mineral oil	1/20	1/10	nd	3
Geraniol	106-24-1	Aldrich	> 97	None	None	1/8	3.8	5
Nerol	106-25-2	Aldrich	> 97	None	None	1/8	2.6	5
Geranyl acetate	16409-44-2	Aldrich	> 98	None	None	1/8	0.4	5
Neryl acetate	141-12-8	Aldrich	> 98	None	None	1/8	0.4	6
<i>trans,trans</i> -2,6-Nonadienal	17587-33-6	Acros	95	Mineral oil	1/20	1/10	nd	3
<i>trans,trans</i> -2,6-Nonadienal	557-48-2	Acros	95	Mineral oil	1/20	1/10	nd	4

¹ CAS#, Chemical Abstract Services registry number; ppm, parts per million in air; nd, not determined.

² Five rats were used in one experiment comparing three bond types, while five different rats were used in another experiment comparing two bond types and two functional groups.

Table 3 Statistical comparisons of factor loadings from principal components analysis of data matrices for single and triple-bonded methyl esters and acids.

2-way ANOVA									
factor	% of variance	bond type		functional group		interaction		P	P
		F	P	F	P	F	P		
1	28.8	286.4	< 0.0001	6.3	0.024	0.6	0.6	0.47	
2	23.2	393.3	< 0.0001	5.8	0.029	1.2	1.2	0.29	
3	7.4	0.5	0.48	84.3	< 0.0001	3.1	3.1	0.1	

Table 4
Statistical comparison of factor loadings from principal components analysis of data matrices for ten-carbon alcohols.

2-way ANOVA								
factor	% of variance	branching		bond type		interaction		P
		F	P	F	P	F	P	
1	25.1	4.4	0.052	0.0	0.94	2.6	0.12	
2	10.3	0.0	0.91	0.9	0.36	0.0	0.94	
3	6.3	9.9	0.006	7.6	0.014	0.0	0.83	

Table 5 Statistical comparison of factor loadings from principal components analysis of data matrices for geranyl and neryl odorants.

2-way ANOVA								
factor	% of variance	functional group		stereochemistry		interaction		P
		F	P	F	P	F	P	
1	37.9	51.8	<0.0001	0.0	0.85	0.6	0.44	
2	7.0	7.8	0.012	0.0	0.96	0.0	0.37	
3	5.7	1.0	0.32	0.8	0.39	0.0	0.91	